Gelatin manipulation of latent macropores formation in brushite cement

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Macroporous brushite cement was prepared from a mixture of β-tricalcium phosphate (β-TCP) and monocalcium phosphate monohydrate (MCPM) using gelatin powder as a latent templates. In a setting reaction coexisting with gelatin, closed packed, open-pore structure with 100–200 μm macropores are obtained after immersion of the set cement into PBS buffer (pH 7.4) at 37 °C for 1–4 weeks. The macroporous brushite cement has compressive strength of 15 MPa originally, which reducing to 5.5 MPa with macropore formation gradually in comparison to that of cancellous bone (5–10 MPa).

1. Introduction

As we know, nature uses small amount of macromolecules to manipulate nucleation, growth microstructure and consequently, the properties of mineral-based materials. Macromolecules applied include proteins, glycoproteins and polysaccharides. Stupp *et al*. utilized poly(amino acid), oligopeptides and synthetic polyelectrolytes as manipulators of mineral microstructure to prepare ''organoapatites''. They were characterized by their improvements in mechanical property and biofunctionality [1].

Following biomimetic concept, there have been many studies on the formation of calcium phosphate cement/polymer composite [2–7], since bone is a natural composite primarily composed of inorganic calcium phosphate and organic fibrous collagen. Bone regeneration requires a suitable scaffold for the growth of bone cells and a viable, well vascularized host bed. Porous scaffold will be of benefit to immediately allow room for bone growth into the construct. Work has shown that the optimal pore range is 200–300 μm with the average human osteon size of ca 223 μm [8]. Pore shape can have a profound effect on the attachment and long-term survival of cell on a surface. For a specific cell type, there is an optimal pore topography that can be readily modulated by careful selection of porogen [9]. However porosity can advesely affect important mechanical properties of a material. So more material research on this respect needs to be carried out.

Gelatin is a denatured derivative of collagen. The amphiprotic macromolecules with isoelectric point being ca 4.5 has a high content of carboxyl groups and suitable amount of amino groups which will lead to interactions between gelatin chains and mineral ions e.g. Ca^{2+} and PO_4^{3-}, respectively.

With regard to bioceramics, brushite cement composed of a mixture of β-TCP and MCPM was chosen as a model mineral to study macropore formation in its set cement by using gelatin as a latent porogen mixed in powder components. The microstructure and properties of the macroporous cement obtained were studied in this paper.

2. Materials and methods

2.1. Materials

The β-TCP powder was prepared by heating a 2:1 molar mixture of dicalcium phosphate dihydrate (DCPD) and calcium carbonate ($CaCO_3$) in air for at least 2 h at 950 °C and subsequent natural cooling. Gelatin was purchased from Sigma Chemical Co., USA. MCPM, tetrasodium pyrophosphate and other chemicals were all analytical reagents and used as supplied.

2.2. The preparation of macroporous calcium phosphate cements

Calcium phosphate cements consisting of β-TCP and MCPM were prepared according to reference [10]. Briefly, a powder mixture of β-TCP and MCPM was mixed with 0–15% of dried gelatin powder. The liquid phases of cements were made through dissolving tetrasodium pyrophosphate (0.2 M) in deionized water. The cement powder phase was mixed with liquid phase to a paste at various powder-to-liquid (P/L) ratios and packed tightly into a 2 ml plastic syringe to form 9 mm D × 12 mm H samples. The cement specimens were stored in a 37 °C, 100% relative humidity incubator for 24 h, and soaked in PBS (pH 7.4) at 37 °C for 1–4 weeks.

2.3. The setting time test

The setting time (ST) of the cement sample was measured according to the method set out in international standard ISO1566 for dental zinc phosphate cements [3].

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In this method, the cement is considered set when a 400 g weight loaded on to a Vicat needle with a tip diameter of 1 mm fails to make a perceptible circular indentation on the surface of the cement.

2.4. Characterization of macroporous cements

The cement compositions were characterized by X-ray diffraction (XRD) for qualitative phase analysis with a BDX3300 diffractometer system (Beijing University Instrument Factory, Beijing, China). Scanning electron microscopy (SEM) observation was carried out for microstructural analysis by using Philips XL30 (Philips-FEI Company, Netherlands).

2.5. Mercury porosimetry

The porosity and mean pore size of set cement samples were determined by mercury porosimetry (Micromeritics Poresizer 9320, USA). Samples of ca 1 g were used in each measurement. The intrusion pressure was ranged from 4.9 KPa to 3.5 MPa and the pore diameter distribution was obtained from the Washborn equation:

$$d = \frac{-4\gamma\cos\theta}{P}$$

where γ is the surface tension of mercury (4.8×10^{-2} N), θ is the advancing contact angle between mercury and specimen (140°), and P is the pressure measured during the intrusion process. The porosity was calculated from total introduced volume (per unit mass, Vi) as following formulation [11]:

$$\varepsilon = \frac{Vi}{Vi + 1/\rho_c}$$

2.6. Compressive strength determination

The compressive strength (CS) of the wet samples were measured by using an M500-10AX testing machine (Testometric Universal Tester, England) at loading rate of 1 mm/min. The CS value obtained were an average value for at least five specimens.

3. Results and discussion

Brushite was a kind of calcium orthophosphate cement reported by Lemaitre *et al*. [12], first. They found that the mixture of MCPM and β-TCP was blended with water to a paste, it has a short setting time. The reaction product is DCPD:

$$\beta\text{-}Ca_3(PO_4)_2 + Ca(H_2PO_4)_2 \cdot H_2O + 7H_2O \rightarrow 4(CaHPO_4 \cdot 2H_2O)$$

The addition of sulfate (SO_4^{2-}), citrate ($C_6H_5O_7^{3-}$), and pyrophosphate ($P_2O_7^{4-}$) increased setting time and mechanical properties [13, 14]. Researchers believed that brushite cements have faster resorption and replacement by bone formation than other apatite cements such as TTCP/DCPA-based one when tested in small animals e.g. rabbits [15, 16]. However, it was also found that when the brushite cements tested were several times larger than those tested in rabbits, cement resorption and new bone formation observed were obviously slow down [17].

3.1. Setting time (ST), porous property and compressive strength (CS)

The influences of gelatin content on the resulting ST, porosity and CS of the β-TCP/MCPM-based cements are summarized in Table I. The ST and porosity increased with enhancing gelatin content. On the contrast, the wet CS value reduced rapidly with the addition of gelatin. The observed relationship between the CS and porosity was similar to that observed for most porous materials, i.e. mechanical strength decreased rapidly with an increase in porosity. SEM micrographs of the fracture surface of the set cements with different amounts of gelatin soaked in PBS for 1 week are shown in Fig. 1. There are larger than 100 μm pores within the samples with gelatin content more than 5%. This result is agreed with the pore diameter distribution curves of the gelatin templated cement in Fig. 2. In recent years, many *in vivo* studies have revealed the significance of the porous structure of the biomaterials on the promotion of bone ingrowth [18–20]. However, Hulbert *et al*. [21] reported that macropores of at least 100 μm are needed to host the cellular and extracellular components of bone and blood vessels, and greater than 200 μm are expected to be effective in osteoconductivity. In this study, raising porosity and enlarging pores resulted in decline of mechanical strength. If bone ingrowth into the macroporous cement were very rapid, the problem may eventually be overcome. Anyway the cement under investigation provides CS whose value corresponding to that of low value of cancellous bone (5–10 MPa). It is worthy to note that the set cement will drop in its

TABLE I Effects of gelatin content on the setting time, compressive strength, and porosity of β-TCP/MCPM-based cements at P/L = 2.7

Gelatin content (%)	ST (min)		CS(MPa)			Porosity (%)	
	n	Mean ± SD	*n*	Mean(SD)*	Mean(SD)†	*n*	Mean(SD)†
0	4	7(1)	5	15.1(1.9)	13.6(0.9)	3	43.2(0.8)
1	4	7(1)	5	13.8(0.7)	12.6(1.7)	3	43.7(0.6)
2	4	8(1)	5	9.8(2.0)	10.3(1.2)	3	44.5(1.2)
5	4	14(2)	5	5.6(0.1)	5.3(0.7)	3	48.7(1.4)
8	4	16(2)	5	5.6(1.0)	5.0(0.6)	3	51.6(1.0)
15	4	19(2)	5	4.0(0.5)	3.7(0.3)	3	54.3(0.5)

*Before soaking in PBS.
†After soaking in PBS for 1 week at 37 °C.

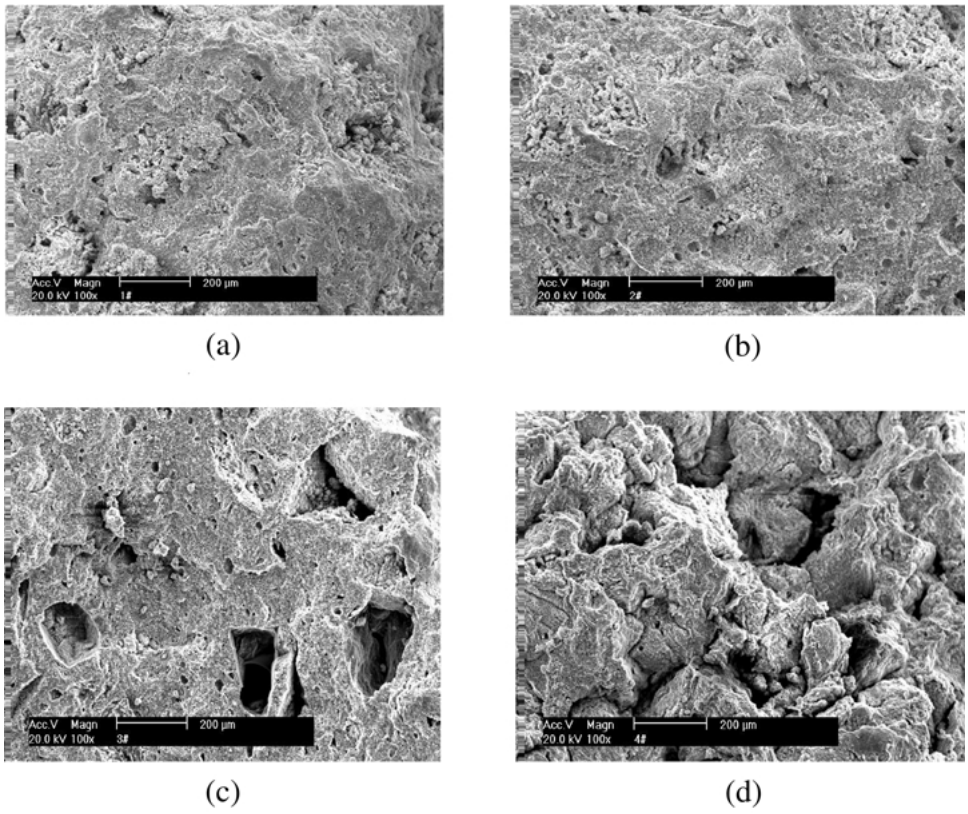

Figure 1 SEM pictures of the fractured surface of cement specimens with gelatin: (a) 0%; (b) 2%; (c) 5%; (d) 15%.

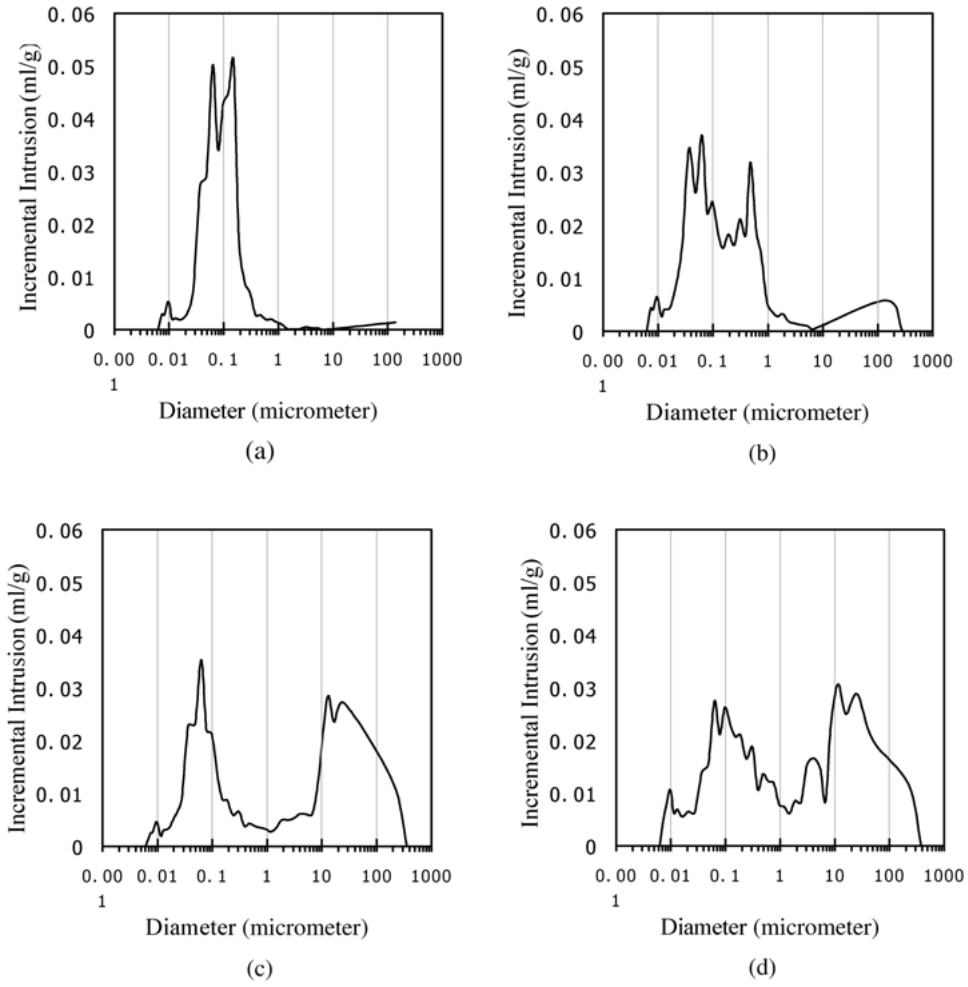

Figure 2 Pore diameter distribution curves of cement specimens with gelatin: (a) 0%; (b) 2%; (c) 5%; (d) 15%.

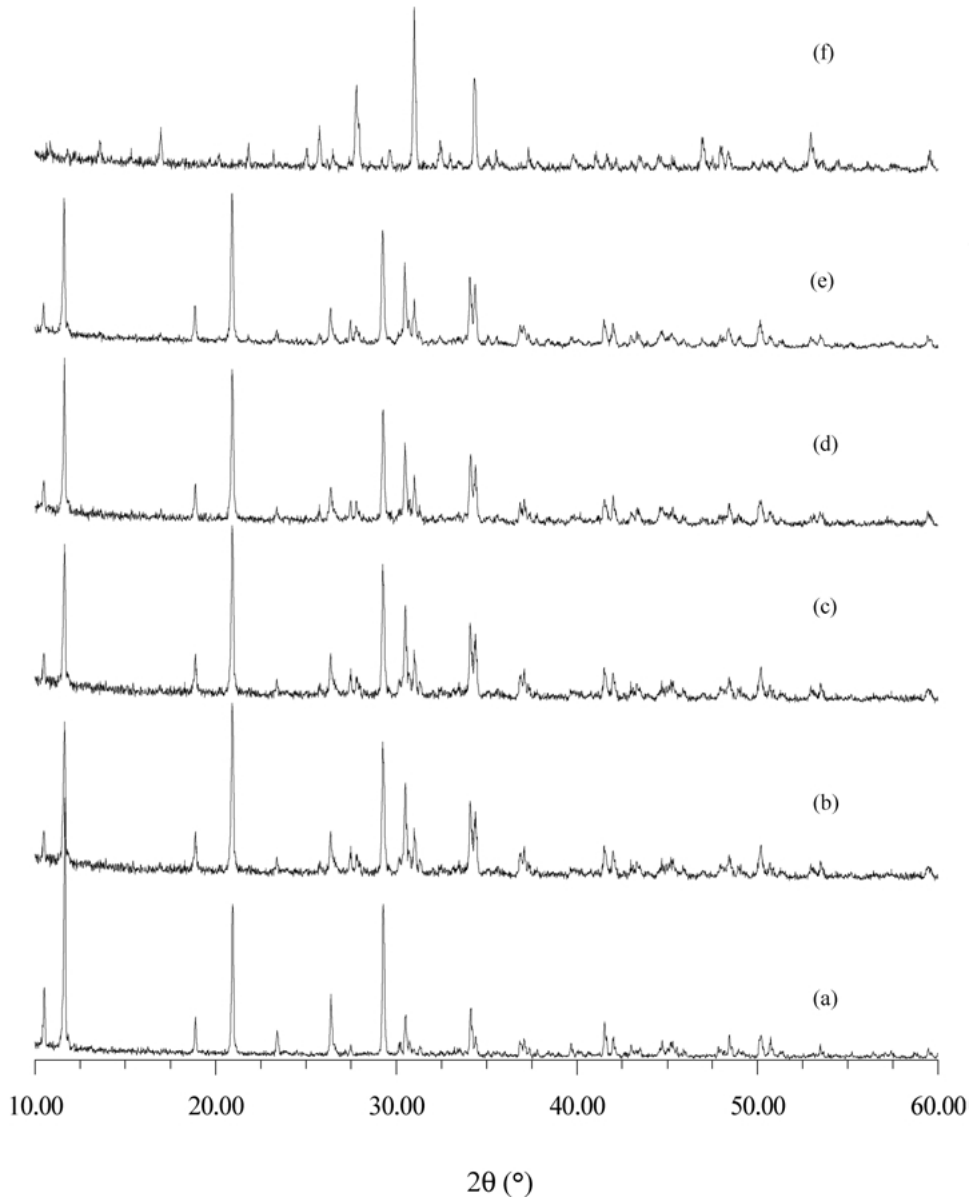

Figure 3 X-ray powder diffraction profiles of DCPD, β-TCP and β-TCP/MCPM cement specimens with gelatin: (a) DCPD; (b) 0%; (c) 2%; (d) 5%; (e) 15%; (f) β-TCP.

compressive strength gradually *in vivo* that will help to the bone regeneration because gelatin is utilized as a latent porogen, the macropores formed with gelatin leaching *in situ*.

3.2. Microstructure analysis

To ascertain the microstructure effects of gelatin on the set cements, XRD analysis was employed and the diffraction patterns of the cement specimens revealed a typical DCPD crystal structure with small amounts of β-TCP as shown in Fig. 3. The *in vivo* absorption of brushite cements was relatively faster than that of apatite cements. In order to slow down the absorption rate, brushite cements were usually prepared with an excess of β-TCP and some amounts of β-TCP sintered granules were added at a sacrifice of mechanical properties [22]. In our case, because of the molar ratio of β-TCP and MCPM was 1.22 that resulted in the existence of β-TCP in the XRD patterns after soaked in PBS for 1 week. On the other hand, a small excess of β-TCP in the initial cement composition can provide relatively high pH value which is desirable for practical use as bone substitute [10].

As indicated in Fig. 3, there is no obvious difference in X-ray diffraction profiles between the set samples of pristine cement and gelatin templated one. However Fig. 4 displayed that the crystal size of DCPD was gradually decreased with an increase in gelatin content and the entanglement of the precipitated DCPD crystals were also reduced obviously. It might be another reason for the descent of CS besides the porosity increasing. In fact the addition of gelatin to the cement also led to a gradual drop of CS before soaking in PBS (cf. Table II). Moreover, the CS of cement sample containing 5% gelatin was almost not varied even soaked in PBS for 1 month, as indicated in Table II. These data imply that the main reason for the decrease of mechanical properties would be due, for the most part, to the reduced interlocking of precipitated DCPD crystals rather than increased porosity by gelatin leaching. It seems that gelatin inhibits the dissolution of β-TCP, MCPM and the diffusion of calcium and phosphate ions because the ST of the cement was extended from 7 to 19 min with the

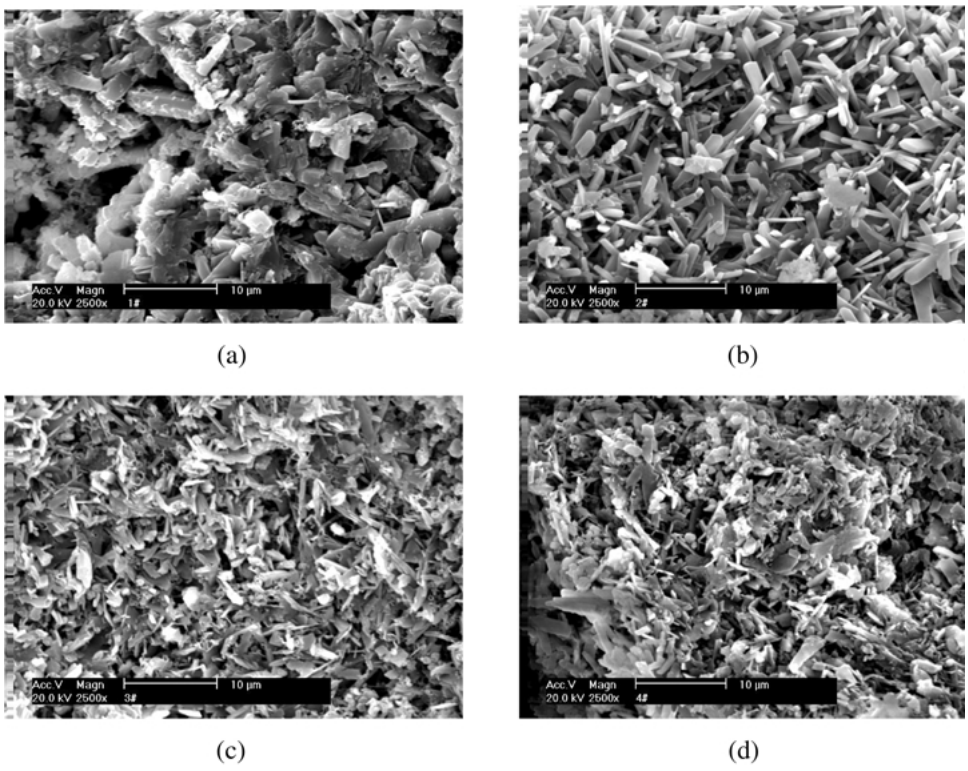

Figure 4 SEM micrographs of the fractured surface of the set cement specimens with gelatin. (a) 0%; (b) 2%; (c) 5%; (d) 15%.

TABLE II Soaking time dependence of compressive strength of β-TCP/MCPM-based cements without gelatin and with 5% gelatin at P/L = 2.7

Soaking time	CS (MPa) Without gelatin		CS (MPa) With 5% gelatin	
	n	Mean(SD)	*n*	Mean(SD)
before soaking	5	15.1(1.9)	5	5.6(0.1)
1 week	5	13.6(0.9)	5	5.3(0.7)
2 weeks	5	13.9(2.0)	5	5.7(0.8)
1 month	5	13.7(1.2)	5	4.7(0.3)

TABLE III Effects of the amount of liquid phase on the setting time, compressive strength and porosity of the β-TCP/MCPM-based cements without gelatin

P/L	ST(min)		CS(MPa)*		Porosity(%)*	
	n	Mean(SD)	*n*	Mean(SD)	*n*	Mean(SD)
1.3	4	26(2)	5	2.7(0.4)	3	53.6(1.4)
1.6	4	20(2)	5	4.2(0.5)	3	49.5(0.5)
2.0	4	12(1)	5	9.9(1.6)	3	47.8(1.6)
2.7	4	7(1)	5	13.6(0.9)	3	43.2(0.8)
2.9	4	6(1)	5	13.5(2.0)	3	43.6(1.0)
3.2	4	3(0.5)	5	13.4(1.2)	3	42.3(1.2)

*After soaking in PBS for 1 week at 37 °C.

addition of 15% gelatin. As stated above, since gelatin macromolecule has plenty of carboxyl, amino and hydroxy groups which could adsorb onto β-TCP and MCPM surface via their electrostatic interaction, hydrogen bonding and coordination complex formation. Gramin *et al*. [23] reported that polymers were able to adsorb onto HAP and possess calcium-chelating power that inhibited dissolution of this mineral.

Cement samples prepared without special additives contain inherent micropores which are primarily a result of the volume taken up by the content liquid. In fact water content was one of the important factors of bone cements and it was usually used to control porosity with some water-soluble salts such as $NaHCO_3$ and Na_2HPO_4 [24, 25]. As shown in Table III, the porosity of the setting cements was gradually increased when much liquid phase was used and the CS reduced more drastically than that of gelatin templated cements did (cf. Table I). However, both morphology and size of the DCPD crystals were almost not affected by the increment in

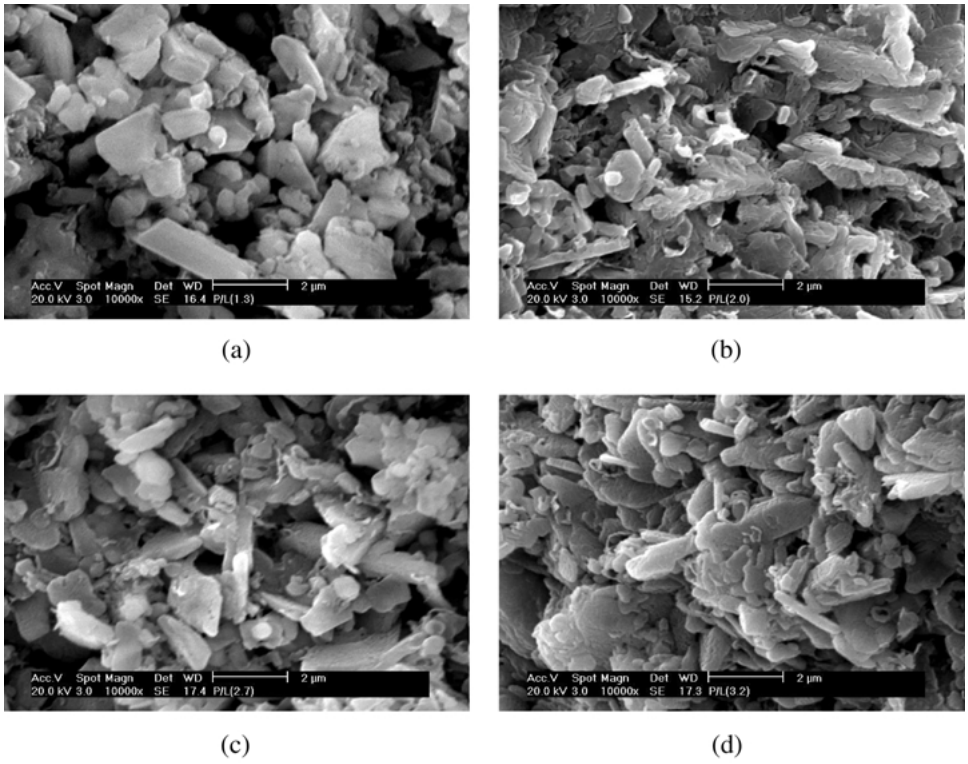

Figure 5 SEM pictures of the fractured surface of the set cement specimens without gelatin at different P/L. (a) 1.3; (b) 1.6; (c) 2.0; (d) 2.7.

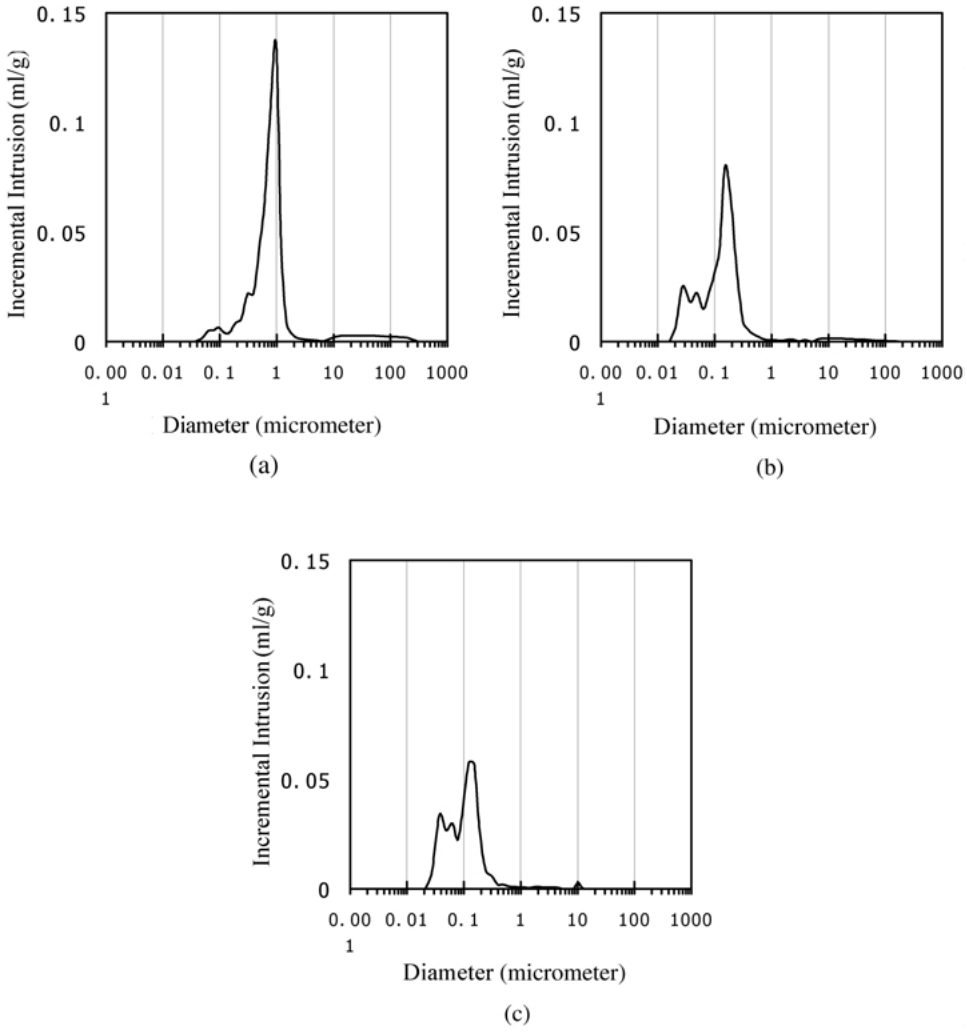

Figure 6 Pore diameter distribution curves of cement specimens without gelatin at different P/L. (a) 1.3; (b) 2.0; (c) 2.7.

liquid phase (Fig. 5). Moreover, as shown in Fig. 6, pores larger than 5 μm are hardly found in these serial cements even their porosity is similar to that gelatin templated ones (cf. Table I and Table III). These results denote the pore formation mechanism with water or gelatin as porogen was completely different.

4. Conclusion

This study elucidated the feasibility of the preparation of macroporous brushite cement via using gelatin as a latent porogen. Its compressive strength is correspond to low value of cancellous bone. Anyway this kind cement would be a promising bone substitute material which would benefit for bone cell ingrowth where more rapid resorption rate and remodeling are highly important.

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